Macromolecular Research, Vol. 13, No. 6, pp 549-552 (2005)

Crystallization and Preliminary X-ray Analysis of Native and Selenomethionyl Polymyxin Resistance Protein D from *E. coli*

Hyesun Jung, Seyoung Han, Taegyun Kim, Mahn-Joo Kim, and Changill Ban*

Department of Chemistry and Division of Molecular and Life Sciences (BK21), Pohang University of Science and Technology, Kyungbuk 790-784, Korea

Received August 29, 2005; Revised October 12, 2005

Introduction

Polymyxin B, a cationic antimicrobial peptide has been isolated from *Paenibacillus polymyxa*. The cationic peptide has polycationic, amphipathic and cyclic unique features.[1] These characteristics of the cationic antimicrobial peptide are thought to contribute to the destruction of gram-negative bacteria by through an initial interaction with the negatively charged surface molecule lipopolysaccharide (LPS), which leads to self promoted uptake across the outer membrane into the negatively charged cytoplasmic membrane of the bacterium.[2] This leads to membrane perturbation and probably translocation of the peptide across the membrane. Some gram-negative bacteria resistant to polymyxin B possess mechanism that modify the LPS through neutralization of its negative charge, which decreases the binding affinity of polymyxin B.[3]

Several of the genes regulated by PhoP/PhoQ and PmrA/PmrB encode enzymes that modify either the acyl chain region or the phosphate group of LPS. In *Salmonella*, polymyxin B resistance is mainly controlled by the PmrA/PmrB two-component system (Figure 1). PmrA-activated genes encode periplasmic and cause the modification of the LPS.[4] Transcription of PmrA-activated genes is not only promoted by high levels of extra-cytoplasmic Fe^{3+}, which is sensed by the PmrB protein, also it is activated by the PhoP/PhoQ system in response to low levels of extra-cytoplasmic Mg^{2+}.[5] For activation of PmrA at the low level Mg^{2+} condition not only requires the noncognate sensor PhoQ protein but also its cognate regulator PhoP, and the PhoP activated PmrD gene.[6] The PmrD protein appears to control the PmrA/PmrB system at a post-transcriptional level. The PmrD protein activates the PmrA/PmrB system via a protection mechanism through dephosphorylation of PmrA.[7] The protective activity of PmrD protein is suggestive of the behavior displayed by certain members of the 14-3-3 protein family in eukaryotes to prevent the phosphorylated state of CDC25.[8]

*Corresponding Author. E-mail: ciban@postech.ac.kr

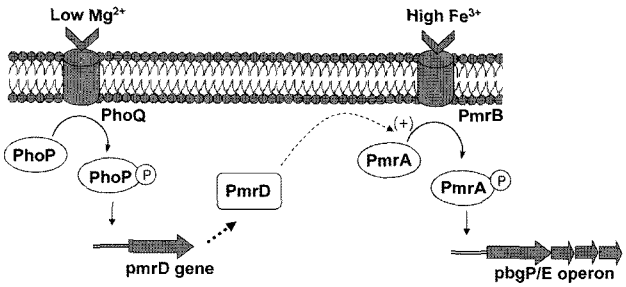


Figure 1. Schematic illustrating the regulatory interactions between the PhoP/PhoQ and PmrA/PmrB two-component systems and the PmrD protein. Transcription of PmrA-activated genes is induced by growth in high iron independently of the PhoP/PhoQ two-component system (right) or by growth in low Mg^{2+} via the PhoP/PhoQ system dependent drift (left). For growing in low levels of extra-cytoplasmic Mg^{2+}, the PhoP/PhoQ system promotes expression of pmrD gene. The PmrD protein may function by protecting of dephosphorylation of PmrA protein in *Salmonella*. Increasing the levels of phosphorylated PmrA protein may result that promote the seven-gene pbgP/E operon.

It has recently been reported that the differential regulation of homologous genes is the mechanism responsible for the divergence of *Salmonella enterica* and *E. coli* in their ability to make LPS modifications and mediate resistance to polymyxin B.[9] In contrast to *Salmonella*, *E. coli* K-12 induces PmrA-activated gene transcription and polymyxin B resistance in response to Fe^{3+}, but it makes no response to low levels of Mg^{2+}.[10] This discrepancy suggests that the structure of *E.coli* PmrD differs from *Salmonella* PmrD. In particular, the N-terminal region of PmrD, which differs between the *E.coli* and *Salmonella* proteins, is in part responsible for the distinct behaviors of the PmrD proteins (Figure 2). In the present report, we describe the crystallization and preliminary X-ray analysis of *E. coli* PmrD.

Experimental and Results

Protein Preparation. The *E. coli* PmrD gene was amplified from K-12 genomic DNA by PCR with appropriate synthetic primers containing BamHI and XhoI restriction sites. An expression vector for His5-PmrD was constructed by inserting the BamHI and XhoI digested fragment of full-length coding DNA for PmrD into pET15b-TEV vector digested with same enzymes. The construct was sequenced by an Automatic DNA Sequencing System.

The His5-PmrD was overexpressed in *E. coli* strain BL21(DE3) with Luria-Bertani broth containing 100 μg/L

```
E.coli       ( 1) MKTLSKQDNAMEWLVKKSCCNKQDNRHVLMLCDAGGAIKMIAEVKSDFAVKVGDLLSPLQ
Salmonella   ( 1) ----------MEWLVKKSHYVKKRACHVLVLCDSGGSLKMIAEANSMILLSPGDILSPLQ

E.coli       (61) NALYCINREKLHTVKVLSASSYSPDEWERQCKVAGKTQ
Salmonella   (51) DAQYCINREKHQTLKIVDARCYSCDEWQR---LTRKPL
```

Figure 2. Comparison of the PmrD protein sequence between *E.coli* and *Salmonella*. Gaps are introduced to optimize alignment.

ampicillin and 0.1 mM IPTG. The His5 tagged protein PmrD was expressed as a soluble protein. The lysate was treated with lysozyme and sonicated in 20 mM phosphate buffer (pH 7.4), 500 mM NaCl, 2 mM β-mercaptoethanol. Cell debris was removed by centrifugation at 22,000 × g at 4 °C, and the resulting supernatant loaded on a Ni-chelating Sepharose fast flow column (Amersham Phamacia). Contaminating proteins and DNA were removed by washing with 20 mM phosphate, 500 mM NaCl, 2 mM β-mercaptoethanol, 45 mM imidazole. PmrD was then eluted in 20 mM phosphate, 500 mM NaCl, 2 mM β-mercaptoethanol, and 300 mM imidazole. The PmrD protein appeared as a major component of the eluted fraction. The protein had an apparent size of 14 kDa as judged by SDS-PAGE (Figure 3). For removal of the His tag, eluted protein was digested with TEV protease and subjected to Ni-NTA affinity chromatography. The untagged protein was eluted by flow through. After protease treatment, the protein had an apparent size of 8.5 kDa as judged by SDS-PAGE (Figure 3). The eluted fractions were concentrated approximately to 1 mL by ultrafiltration with YM-10 membrane, and loaded on a HL Superdex 75 column (Amersham Phamacia). The running buffer for the gel filtration column was 20 mM Tris, 150 mM NaCl, 0.5 mM DTT, 0.5 mM EDTA (pH 7.0). The PmrD was eluted at 78 mL. The resultant peak fractions were concentrated to 12 mg/mL by ultrafiltration (Amicon) with YM-10 membrane before proceeding with the crystallization experiment.

The SeMet-substituted protein was expressed in methionine auxotrophic *E. coli* B834 (DE3) strain. The purification of the SeMet-substituted protein followed the same procedure and condition but included 5 times of antioxidant. The purified proteins constituted about 95% total protein as determined on Coomassie Brilliant Blue stained 18% SDS-polyacrylamide gel (Figure 3).

Crystallization. The crystallization trials were performed using the hanging-drop vapor-diffusion method at 20 °C. The initial crystals were obtained in 2.0 M ammonium sulfate and 50 mM sodium acetate (pH 4.6). Efforts to optimize the initial crystallization conditions were most successful and crystals were obtained that were suitable for X-ray diffraction analysis. The crystals were obtained by mixing equal volumes of the protein solution and a precipitating solution

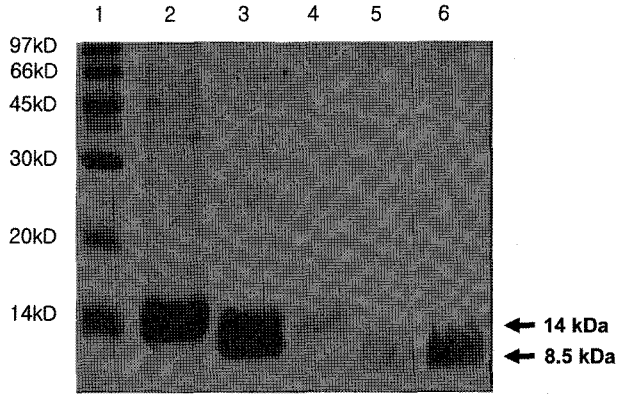
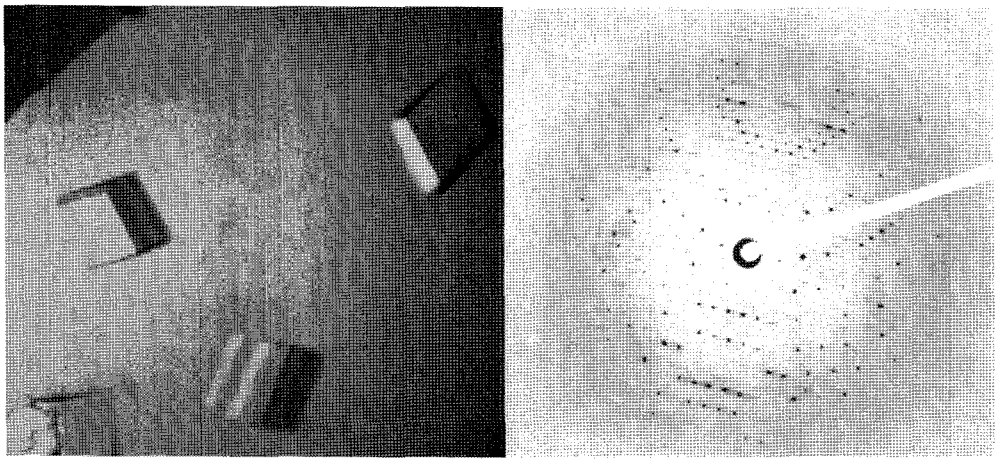


Figure 3. 18% SDS-PAGE shows the purity of *E. coli* PmrD protein. Lane 1, molecular weight markers (all values are in kiloDaltons); Lane 2, eluted fraction from Ni affinity chromatography (The protein had an apparent size of 14 kDa); Lane 3, protein mixture after TEV treatment; Lane 4, eluted fraction at second Ni affinity chromatography (This fraction contains uncut PmrD and TEV protease); Lane 5, Flow-through fraction after second Ni affinity chromatography; Lane 6, concentrated purified PmrD protein after size exclusion chromatography (the untagged PmrD protein had an apparent size of 8.5 kDa).

containing 2.7 M ammonium sulfate and 50 mM sodium acetate (Figure 4(a)). The crystals grew in a week to the maximum dimensions of 0.35 × 0.35 × 0.2 mm^3. The SeMet-substituted protein crystallized well using the same condition for the native protein.

Data Collection and Processing. The crystals were transferred to a cryo-protectant solution (reservoir solution with an additional 20% v/v ethylene glycol), before crystals were picked up with a fiber loop and flash frozen in a stream of nitrogen gas at 100 K.

Diffraction data were collected from 4A beamline at the synchrotron, Pohang Accelerator Laboratory, Pohang, Korea with an ADSC Quantum210 CCD camera as detector, and the Oxford Cryo-system for cooling the sample. The data set was captured using one crystal to detector distance of 180 mm and an oscillation range of 360° in 1° steps. Data, collected at 100 K, were processed using the program HKL 2000. A complete native data set to 2.5 Å was collected on a CCD detector (Figure 4(b)). To solve the heavy atom phas-



(a) (b)

Figure 4. (a) Crystals of SeMet-substituted PmrD grown in 2.7 M ammonium sulfate, 50 mM sodium acetate, pH 4.6. Typical dimensions of crystals were $0.35 \times 0.35 \times 0.2$ mm^3; (b) X-ray diffraction image obtained from a Crystal of SeMet-substituted PmrD. The diffraction limit occurs at 2.7 Å.

ing, multi-wavelength x-ray diffraction data to 2.7 Å were collected from crystals of the SeMet-substituted protein and processed using the program HKL 2000.[11] Auto-indexing yielded unit-cell parameters of a=b=82.96 Å, c=76.16 Å, $\alpha=\beta=90°$, $\gamma=120°$ in the rhombohedral point group R32. Assuming that the crystal has two monomers in the asymmetric unit, a crystal volume per protein mass (Vm) is 3.21 Å^{3}Da^{-1} and the solvent content is approximately 62%.[12] These values are well within the normal ranges observed for protein crystals. The data collection statistics are given in Table I.

Discussion

The difference in ability to execute the PmrA-controlled modification of LPS between *E. coli* and *Salmonella* may contribute to the differential survival of these two enteric species in host and non-host environments. The *E. coli* PmrD protein is only 53.4% identical to the *Salmonella* PmrD protein (Figure 2). We are endeavoring to validate

Table I. MAD Data-Collection Statistics of SeMet-Substituted PmrD

	Low-Energy Remote	Inflection	Peak	High-Energy Remote
X-ray wavelength (Å)	1.12714	0.98178	0.98163	0.97394
Space group	R32	R32	R32	R32
Unit-cell parameter				
a=b (Å)	82.96	82.89	82.83	82.95
c (Å)	76.16	76.01	75.92	76.08
Resolution limit (Å)	2.5	2.7	2.7	2.7
R_{merge} [a] (%)	7.9	6.5	7.1	7.9
Total reflections	69269	56836	57271	58589
Unique reflections	3529	2859	2859	2855
Completeness	97.5	99.5	99.5	99.7
I/σ	16.7	18.0	18.6	16.5
$\Delta f'$ [b]	-1.8097	-5.0593	-5.0084	-3.7001
$\Delta f''$ [c]	6.4744	3.7665	3.7675	3.7072

[a] $R_{merge} = \Sigma_{hkl}\Sigma_i|I(hkl)_i - \langle I(hkl)\rangle|\Sigma_{hkl}I(hkl)$, where $I(hkl)_i$ is the ith measurement of the intensity of reflection hkl and $\langle I(hkl)\rangle$ is the mean intensity of reflection hkl. [b] The imaginary scattering component is proportional to these directly measurable quantities.
[c] The real scattering component f' is related to f'' via the *Kramers-Kronig* relationship.

that the structural difference between PmrD, a connector in the PhoP/PhoQ and PmrA/PmrB systems, is a regulatory difference between *E. coli* and *Salmonella*. The crystals, which were made of SeMet-substituted *E.coli* PmrD, have been obtained with a view to phasing by multi-wavelength anomalous diffraction (MAD).[13] As the 104-residue sequence of *E. coli* PmrD contains 4 methionines, there are 8 Se sites in the asymmetric unit of the SeMet crystal. The Se sites were determined with SOLVE/RESOLVE.[14] Further model building and structure refinement are currently in progress. And we will make every effort to obtain crystals of *S. typhimurium* PmrD.

Acknowledgements. This work was supported by the the KOSEF through Center for Integrated Molecular Systems at POSTECH and the Ministry of Health and Welfare (Grant 02PJ3-TG6-EV05-0001). We thank Dr. K. J. Kim (Pohang Accelerator Laboratory) for technical assistance.

References

(1) R. E. W. Hancock and D. S. Chapple, *Antimicrob. Agents Chemother.*, **43**, 1317 (1999).
(2) M. Wu, E. Maier, R. Benz, and R. E. W. Hancock, *Biochemistry*, **38**, 7235 (1999).
(3) S. D. Breazeale, A. A. Ribeiro, and C. R. H. Raetz, *J. Biol. Chem.*, **277**, 2886 (2002).
(4) J. S. Gunn, K. B. Lim, J. Krueger, K. Kim, L. Guo, M. Hackett, and S. I. Miller, *Mol. Microbiol.*, **27**, 1171 (1998).
(5) M. M. Wosten, L. F. Kox, S. Chamnongpol, F. C. Soncin, and E. A. Groisman, *Cell*, **103**, 113 (2000).
(6) L. K. Kox, M. M. Wosten, and E. A. Groisman, *EMBO J.*, **19**, 1861 (2000).
(7) A. Kato and E. A. Groisman, *Gene & Dev.*, **18**, 2302 (2004).
(8) C. Y. Peng, P. R. Graves, R. S. Thoma, Z. Wu, A. S. Shaw, and H. Piwnica-Worms, *Science*, **277**, 1501 (1997).
(9) (a) F. R. Blattner, G. Plunkett, C. A. Bloch, N. T. Perna, V. Burland, M. Riley, J. Collado-Vides, J. D. Glasner, C. K. Rode, and G. F. Mayhew, *Science*, **277**, 1453 (1997). (b) M. McClelland, K. E. Sanderson, J. Spieth, S. W. Clifton, P. Latreille, L. Courtney, S. Porwollik, J. Ali, M. Dante, and F. Du, *Nature*, **413**, 852 (2001).
(10) M. D. Winfield and E. A. Groisman, *Proc. Natl. Acad. Sci.*, **101**, 17162 (2004).
(11) Z. Otwinowski and W. Minor, *Methods Enzymol.*, **276**, 307 (1997).
(12) B. W. Matthews, *J. Mol. Biol.*, **33**, 491 (1968).
(13) (a) W. A. Hendrickson, *Science*, **254**, 51 (1991). (b) S. Doublie, *Methods Enzymol.*, **276**, 523 (1997).
(14) (a) T. C. Terwilliger and J. Berendzen, *Acta Cryst.*, **D55**, 849 (1999). (b) T. C. Terwilliger, *Acta Cryst.*, **D56**, 965 (2000).